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13. ABSTRACT *(Maximum 200*

Our research is structured to investigate the role of polychlorinated biphenyls (PCBs) in breast cancer induction. Progress is reported in several areas. 1) Higher chlorinated PCBs (#77 + 153) i. induce cytochromes P-450, ii. increase in GST activity, iii. decrease catalase activity, and iv. decrease GSH levels in the livers of treated animals. 2) High concentrations of PCBs were found to cause significant increases in lipid peroxidation and oxidative stress in the livers of treated rats. 3) Mammary tissue levels of oxidized DNA bases suggest a differential response of oxidative stress in PCB-exposed rats. 4) Our measurements of antioxidant enzyme activities in human breast tissue cytosols show enormous inter-individual variations in activities. 5) Structure-activity relationships for PCB metabolites have been described for superoxide production, DNA strand break activity, and cytotoxicity (in several systems including MCF-7 human breast cancer cells). 6) Preliminary studies have been undertaken to react PCB metabolites with DNA bases and to develop the appropriate HPLC conditions to isolate and characterize the reaction products.

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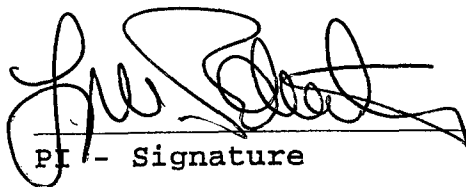
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INTRODUCTION

Our research is aimed at the investigation of the effects of polychlorinated biphenyls (PCBs) in breast cancer. PCBs are industrial chemicals which persist in our environment. The lipophilicity of PCBs and their tendency to bioaccumulate in adipose tissue and breast milk raise concern about the health risks associated with exposure to PCBs and related compounds. Commercial PCB mixtures are complete carcinogens, producing hepatocellular carcinomas in rats and mice, but the mechanisms by which they do so have not been determined. We and others have shown that higher halogenated PCBs (especially, tetra-, penta-, and hexa-chlorinated biphenyls) act as promoters of carcinogenesis, but their initiating or DNA damaging activity has not been conclusively demonstrated. In our original proposal we presented considerable data to support the concept that the lower halogenated biphenyls may be activated by hepatic and breast (milk) enzymes to oxygenated species that are electrophilic and bind to DNA. Of particular interest were the quinone metabolites. Our data showed that PCBs are metabolized to dihydroxy metabolites, that these can be oxidized by peroxidases (including lactoperoxidase) and prostaglandin synthase to quinones, that these PCB-quinones are strong electrophiles that react with both sulfur and nitrogen nucleophiles, including nucleotides and DNA. We offered preliminary observations that supported the concept that PCB quinones redox cycle.

To build on these observations, we proposed (i) to determine if PCBs that accumulate in breast tissue are converted to dihydroxy metabolites that can be oxidized by breast tissue subcellular fractions and lactoperoxidase to PCB quinones, (ii) to characterize the DNA-adducts of breast-specific PCBs with regard to the specific metabolites formed and nucleotides involved and to identify the chemical structure of the adducts, (iii) to determine the biological consequences of DNA-adduct formation by PCB metabolites, including detection of single- and double-strand breaks, analysis of sites that block in vitro DNA synthesis and analysis of mutations, and (iv) to employ in vivo models to identify PCB adducts and mutations in the breast, and to investigate the possibility of using DNA adduction for human biomonitoring purposes by detecting DNA-reacting metabolites in serum and breast milk. These studies address our working hypothesis that PCB congeners that accumulate in the breast may be metabolized in this tissue to electrophiles, especially quinones, which then react with critical cellular targets, including DNA, and that these reactions lead to mutagenic events resulting in neoplastic change. Our project therefore addresses the question of the possible mechanisms of PCB carcinogenicity, with emphasis on the human breast as target organ.

BODY OF THE REPORT

1. EFFECTS OF DI- AND TRICHLORO-BIPHENYLS ON OXIDATIVE AND ANTIOXIDANT ENZYMES IN THE RAT (STUDIES IN THE LIVER)

Although Polychlorinated Biphenyls (PCBs) have been banned from production in many countries since the early 1970s, they still persist in our environment. In fact, PCBs have invaded the food chain, and are bioaccumulating in every organism on the planet. These PCBs accumulate in target tissues such as adipose and breast tissues. Experiments from our laboratory demonstrate that PCBs may be metabolized to dihydroxy metabolites, and that these metabolites may then autoxidize or maybe enzymatically oxidized to semiquinones and/or
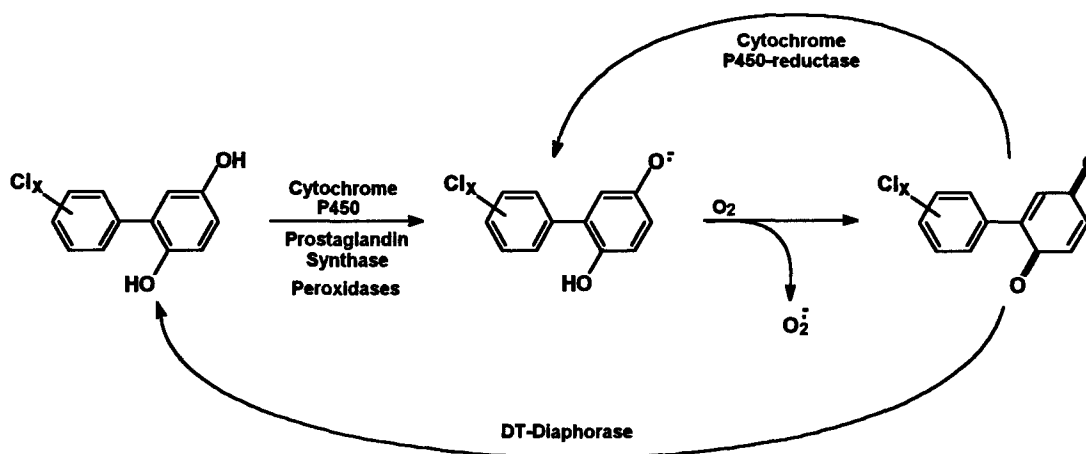
quinones (McLean et. al. 1996). Some of these PCB-quinones can undergo redox cycling, with superoxide being produced from this cycling of metabolites.

PCB-quinones can react with glutathione (GSH) either spontaneously or catalyzed by glutathione transferases. The result is a dihydroxy-PCB-glutathione conjugat. Theoretically the PCB-GSH conjugate is now more readily excreted. But, because a GSH conjugate has been produced doesn't imply that the chance of oxidative damage is decreased. The conjugates can again oxidize enzymatically or non-enzymatically to the PCB-quinone-glutathione conjugate. These PCB quinone-GSH conjugates can be reduced, which indicates that GSH conjugation doesn't prevent quinones from being acted on by DT-diaphorase, or carbonyl reductases. Thus, redox cycling occurs when the hydroxyl and/or hydroxyl-glutathionyl derivatives undergo autooxidation to semiquinone and quinone derivatives with the production of superoxide.

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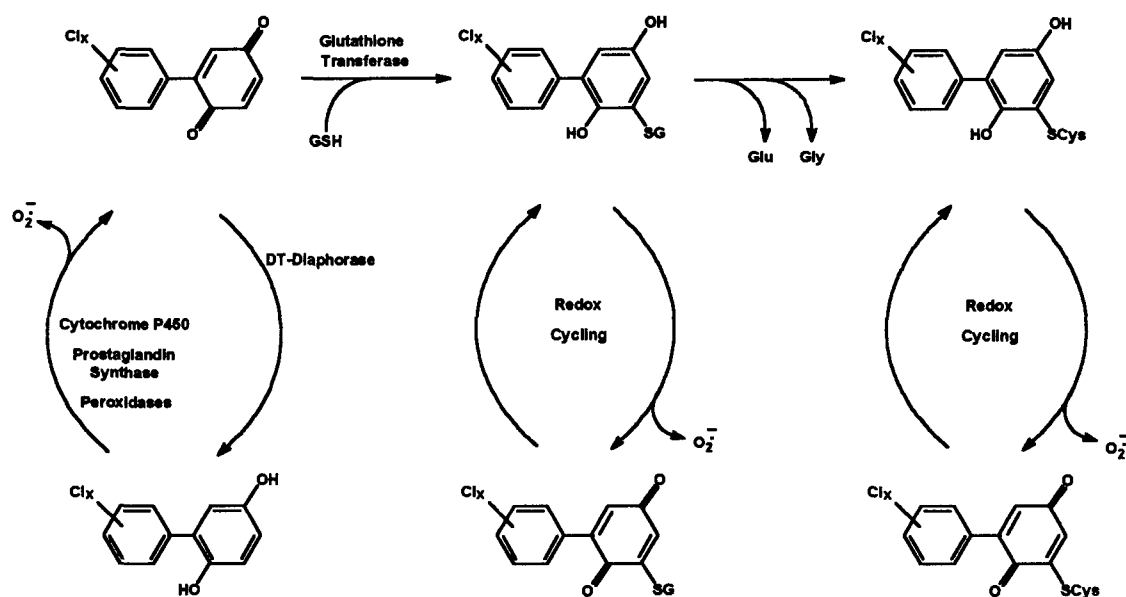
Superoxide is not a very reactive free radical; rather its toxicity rests in its ability to be converted to species that are more reactive. Superoxide dismutase dismutates superoxide to oxygen (O_2) and hydrogen peroxide (H_2O_2) which, in the presence of transition metals, is converted to .OH and –OH. Other detoxifying enzymes in the body are catalase and glutathione peroxidase. Catalase converts H_2O_2 to H_2O and O_2. Glutathione peroxidase couples the reduction of H_2O_2 to the oxidation of GSH. Oxidized GSH can then be reduced by glutathione reductase, which uses NADPH as a cofactor.
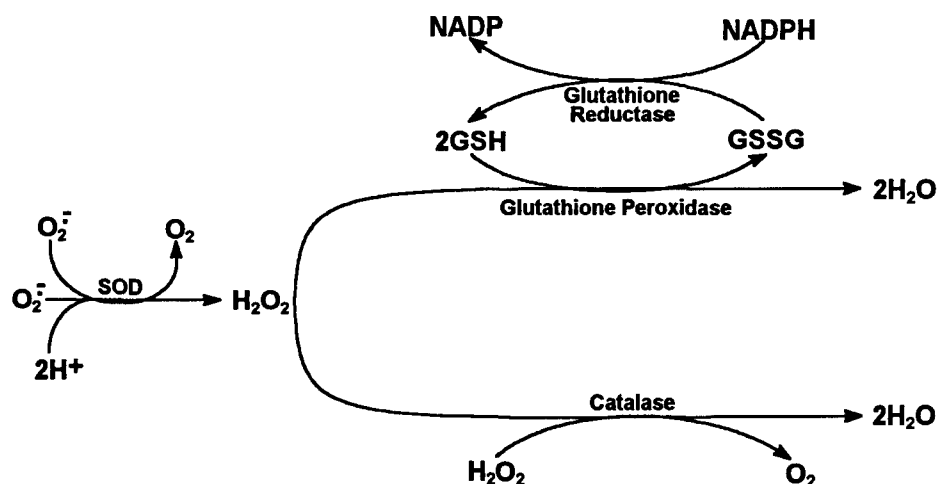
The following study was set up to analyze the effects of PCBs on oxidizing (Cytochrome 450s) and antioxidant enzymes (GSH transferase, GSH peroxidase, catalase) in normal rats and rats that were pretreated with enzyme inducers. We hypothesized that PCB metabolism would occur. The PCB metabolism products would then redox cycle producing superoxide and

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subsequent reactive oxygen species (ROS), thus promoting oxidative damage to DNA. This was to be determined via measurement of 8-oxo-deoxy-guanosine (see next section).

Female Sprague-Dawley rats were treated with corn oil, or phenobarbital+ß-naphthoflavone (PB+ß-NF) or a PCB mixture of PCB 77 (3,3',4,4'-tetrachlorobiphenyl) and PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) for 3 days to induce metabolizing enzymes. This was followed by 3 daily injections of 3,4-dichlorobiphenyl (DCBP) or 3,4,5-trichlorobiphenyl (TCBP) or vehicle alone. The animals were sacrificed and liver and breast tissue removed. The following enzyme and cofactor determinations were performed with liver tissue.

Resorufin assays were performed to show increased P450 enzyme activity, which would result in an increased ability to metabolize PCBs. Animals pretreated with PB+ß-NF as well as animals pretreated with the PCB mixture showed significant increases in activity levels of P450 2B, which was measured using the pentoxyresorufin O-dealkylase (PROD) assay (data not shown). Activity levels of P450 1A2 were also significantly increasaed in PB/ß-NF and PCBs pretreated animals, as determined with the methoxyresorufin O-dealkylase (MROD) assay (see following figure). Finally, using the ethoxyresorufin O-dealkylase (EROD) assay we found that the activity levels of P450 1A1 were also significantly increased in PB/ß-NF and PCBs pretreated animals (see figure).

Glutathione transferase (GST) catalyzes the reaction of an electrophile with reduced glutathione (GSH); GST is not always necessary for the formation of the GSH conjugate. This may also occur spontaneously, but at a slower rate. It is widely accepted that a correlation exists between the activity of P450 1A1 and GST, an increase in P450 1A1 activity normally equates with an increase in GST activity. This is because the GST gene has a dioxin response element in its promoter region, which is activated by the Ah receptor complex, which is also responsible for the induction of CYP 1A1. In agreement with this we observed in our experiment that the GST activity was significantly increased in all animals that were pretreated with PB/ß-NF or the PCB mixture (see figure).

One result of increased GST activity or/and oxidative stress can be a reduction in the GSH level in cells and organs. We found a noticeable decrease in total glutathione in animals pretreated with PB/ß-NF and a significant decrease in total glutathione in PCB pretreated animals (see figure). This is in agreement with previous data from our laboratory where we found that rats treated with only PCB 77, which is a substrate for the Ah receptor, had an increased level of P450 1A1 activity, an increased GST activity level, and a decreased level of total glutathione.

Oxidative stress can lead to an increase in oxidized glutathione (GSSG). GSSG is reduced by glutathione reductase to GSH. We expected that the decreased amounts of total glutathione or increased levels of GSSG due to oxidative stress would result in an increase of glutathione reductase activity in an efford to maintain levels of GSH. As expected, we saw a significant increase in

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glutathione reductase activity in animals pretreated with PB/ß-NF or PCBs (see figure).

Glutathione peroxidase (GPx) is an enzyme that couples the reductive detoxification of H_2O_2 to the oxidation of GSH. It would therefore make sense, if GPx levels were increased during oxidative stress. Otherwise, GPx is a suicide enzyme. Increased production of H_2O_2 may therefore result in decreased GPx levels in organs. No significant decrease or increase in GPx activcity was seen in any of our test groups, however (see figure).

The second enzyme that detoxifies H_2O_2 is the cytosolic enzyme catalase, which reduces H_2O_2 to H_2O and O_2 without a cofactor. An increased level of H_2O_2 could be reflected in an increased catalase enzyme activity level. In our experiment the catalase enzyme activity showed the general trend of being significantly decreased in animals pretreated with PB/ß-NF or the PCB mixture (see figure).

These results show that higher chlorinated PCBs (#77+153) *i.* induce cytochromes P450s, *ii.* result in an increase in GST activity, *iii.* lead to a decrease in catalase activity levels, and *iv.* result in decreased GSH levels. These changes may be the result and/or the reason for increased oxidative stress. These data were obtained from the liver samples of the treated animals. Analysis of these parameters in breast tissue is in progress.

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Ethoxyresorufin O-dealkylase
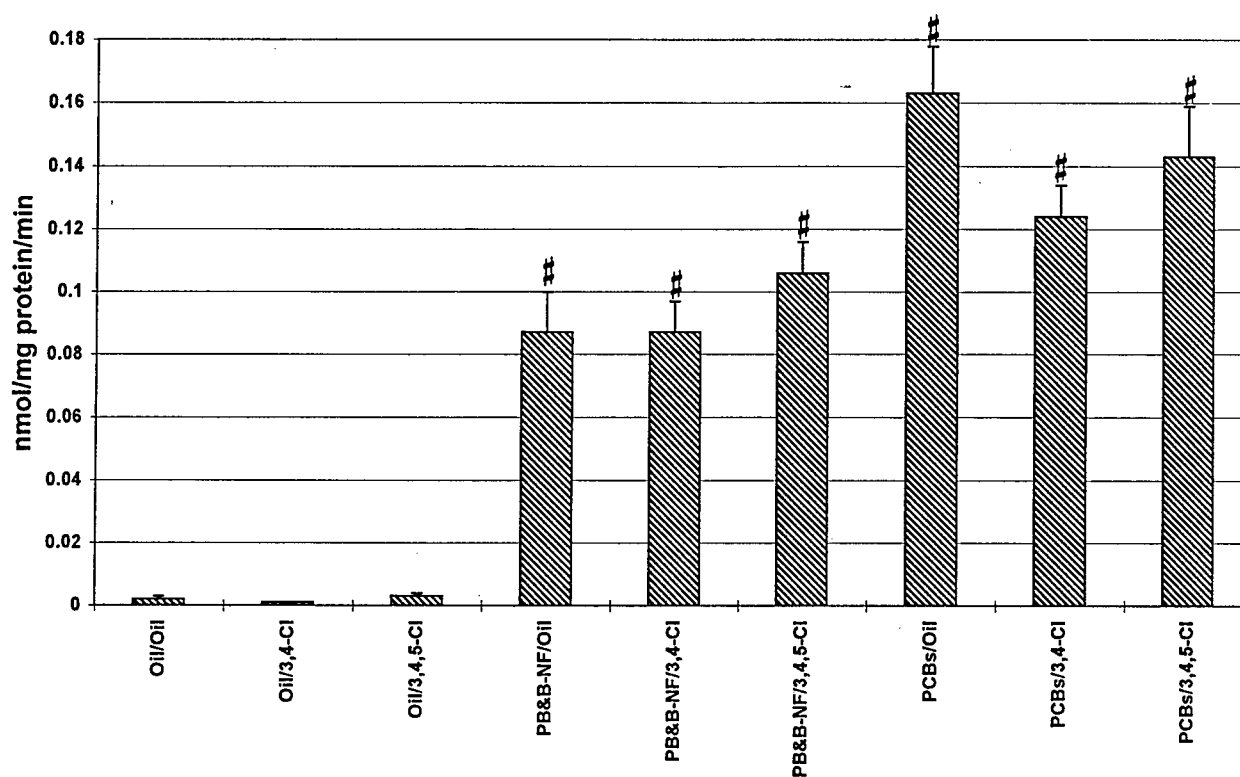
Methoxyresorufin O-dealkylase
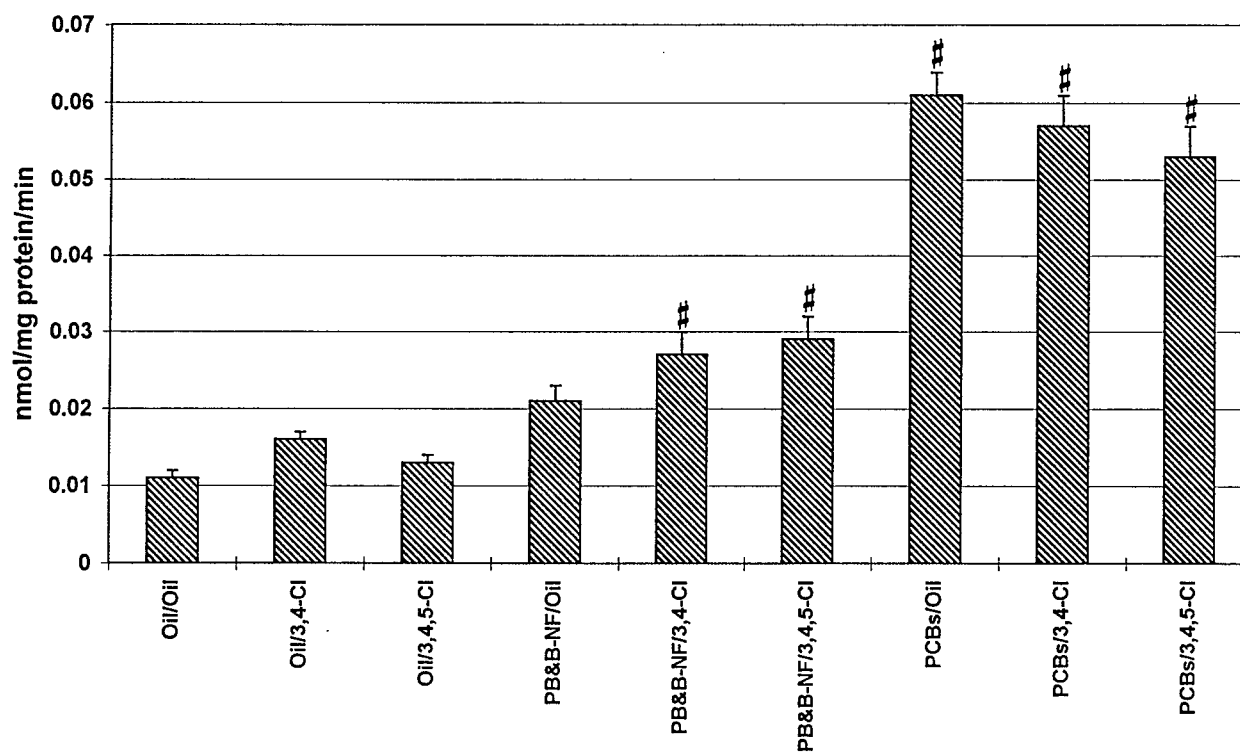
Total Glutathione

Glutathione Transferase

Selenium-Dependent Glutathione Peroxidase

Selenium-Independent Glutathione Peroxidase

Catalase

GSSG Reductase
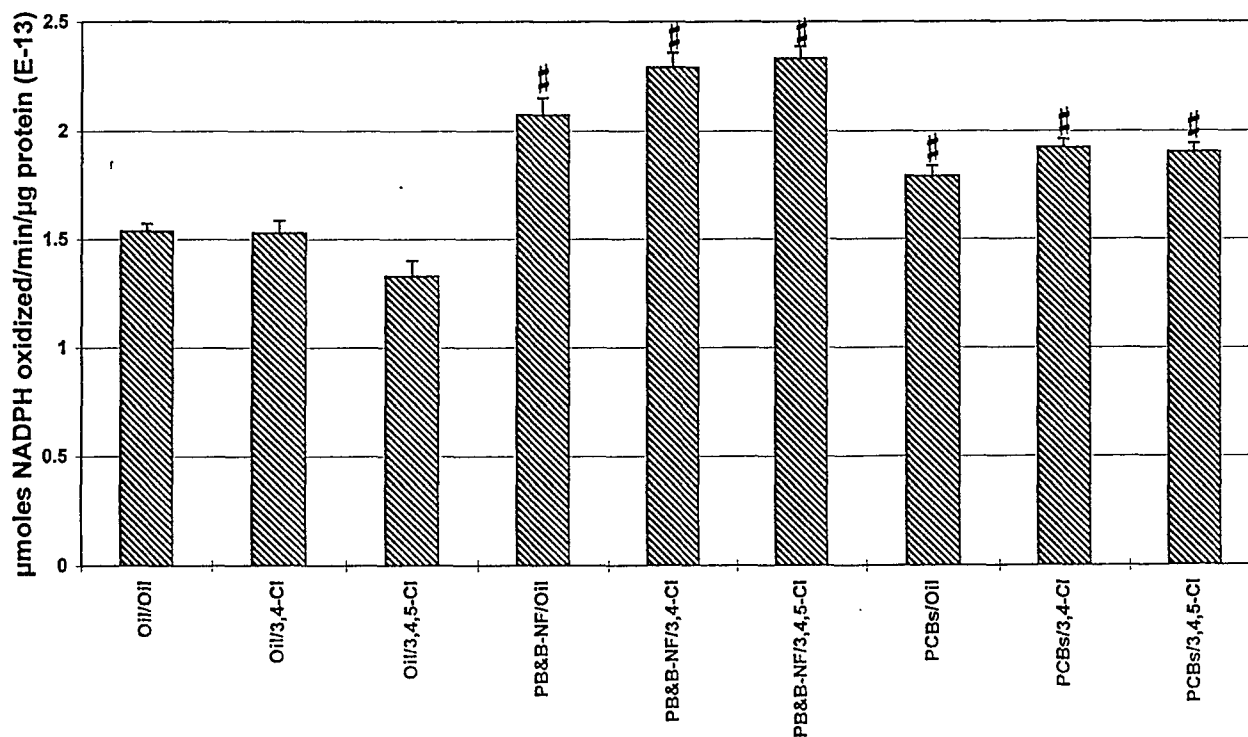
2. EFFECT OF PCBs ADMINISTRATION ON HEPATIC WHOLE HOMOGENATE LIPID PEROXIDATION
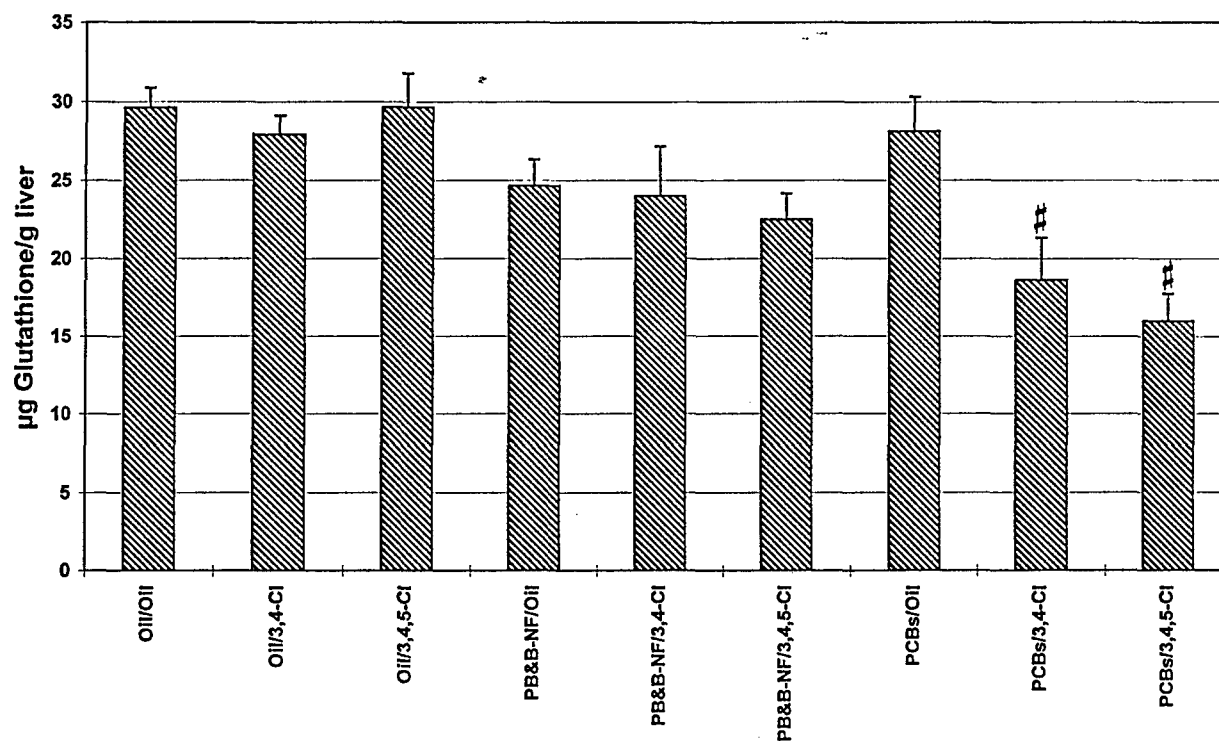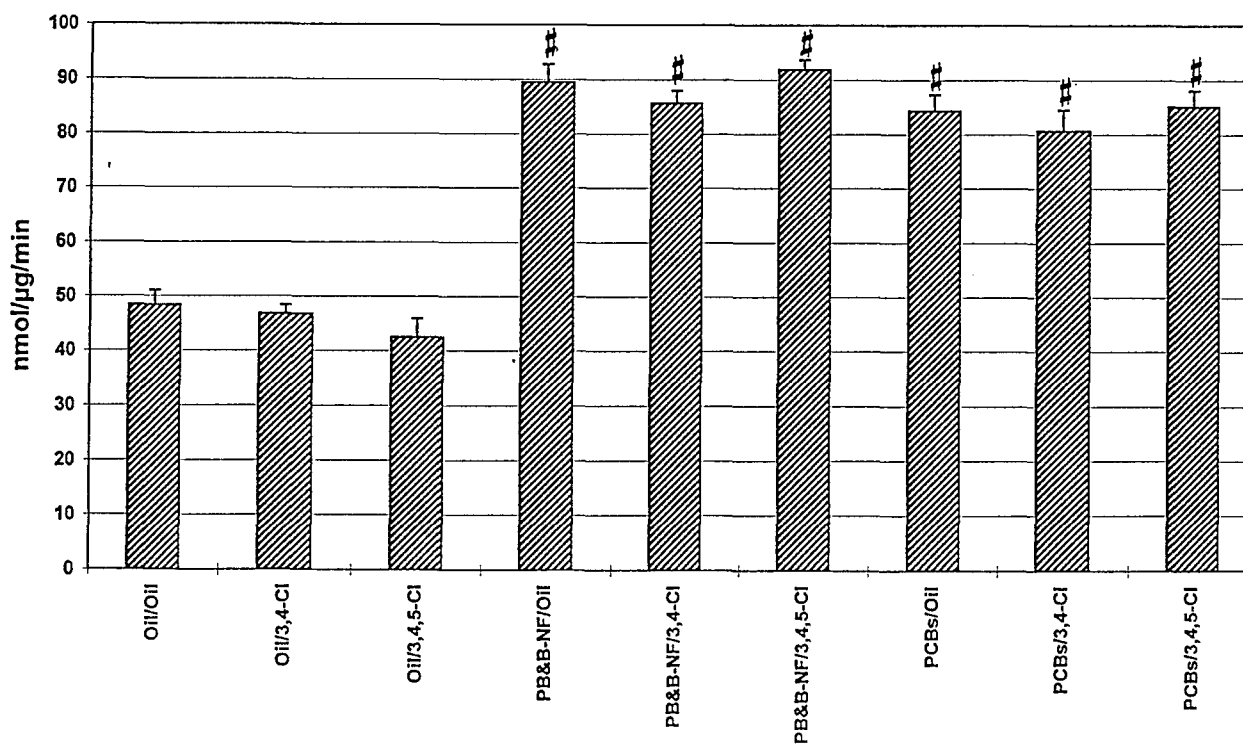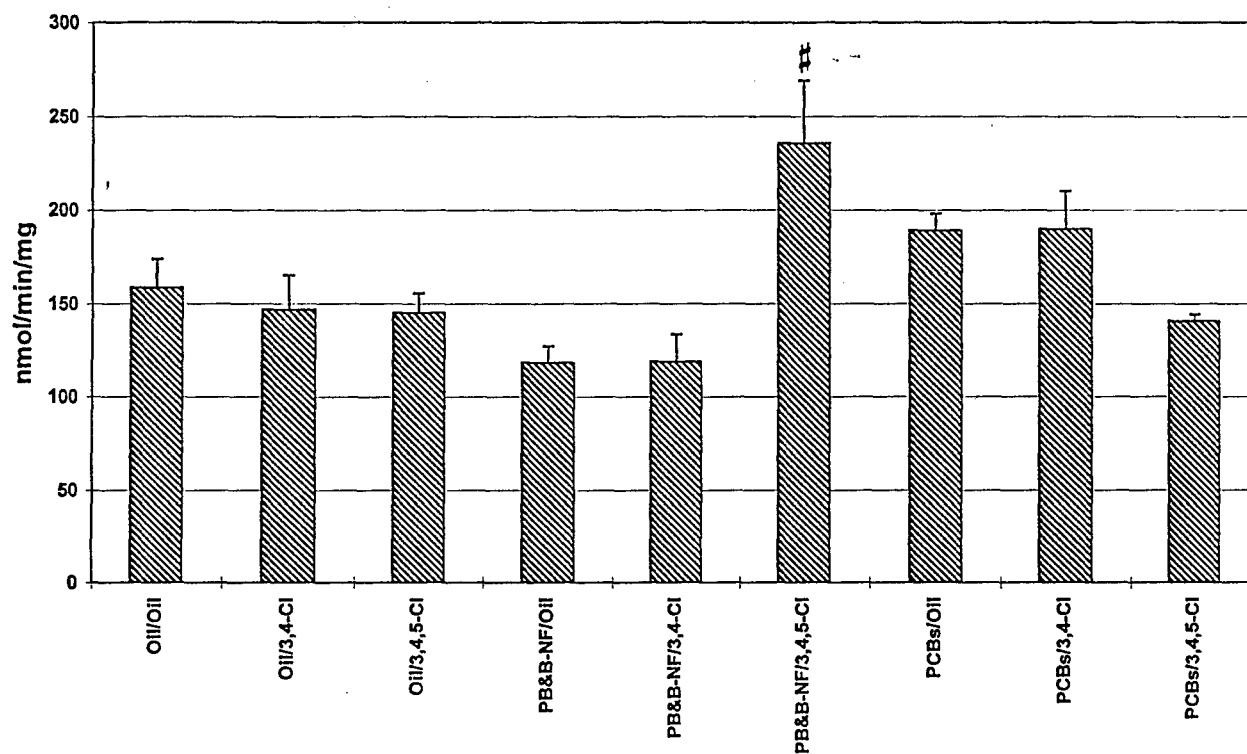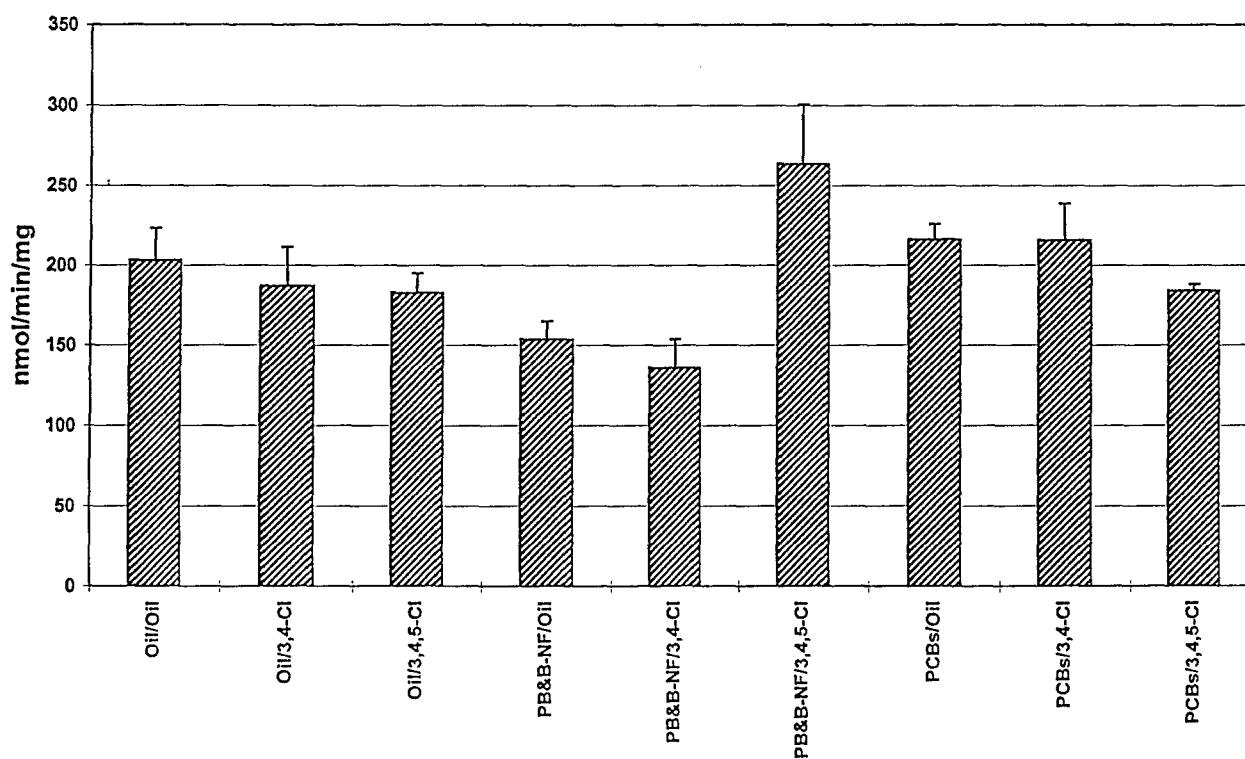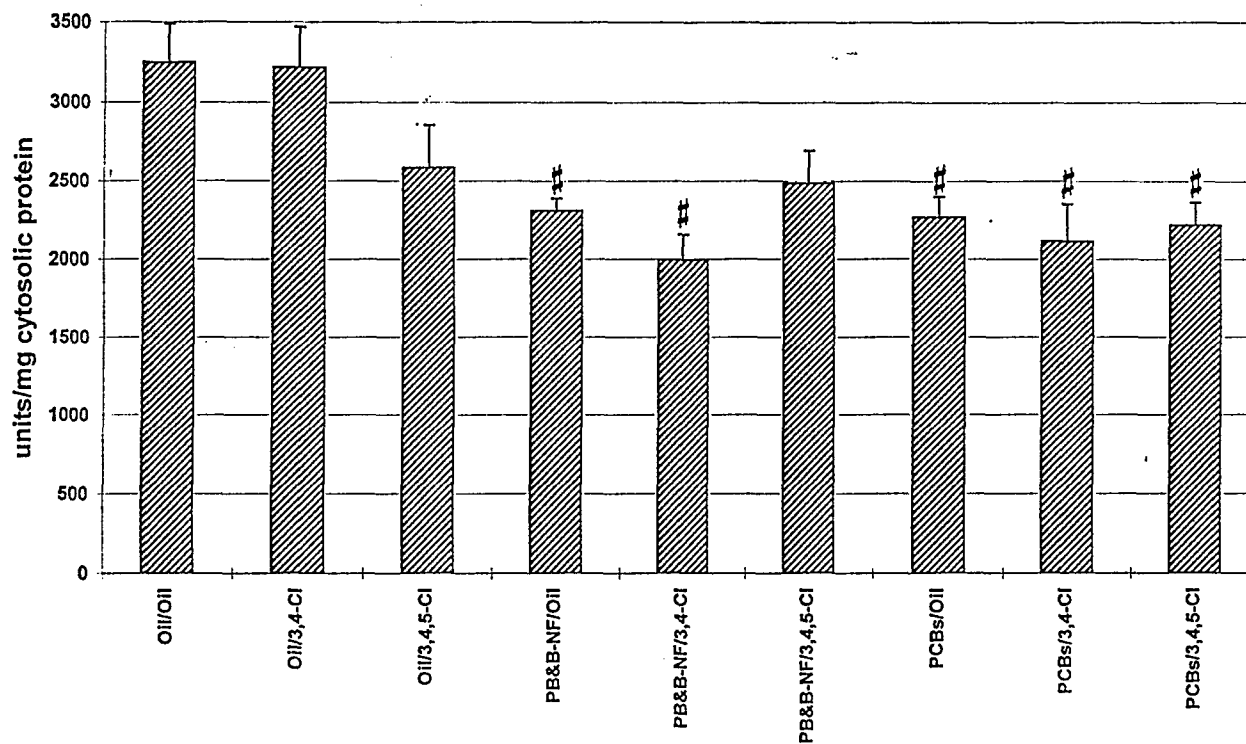
The following study was initiated to study the production of reactive oxygen species as a result of PCBs exposure. The liver was used as a surrogate since sufficient breast tissue could not be obtained from rats for these studies. Furthermore these reactive oxygen species may travel to distant locations, if not inactivated by antioxidant enzymes. Rats were treated with PCB compounds as follows: Each rat received by IP injection, dissolved in corn oil, either 3,3',4,4'-tetrachlorobiphenyl (TCB-77) (at 30, 100, 150 or 300 µmol/kg), 2,2',4,4',5,5'-hexachlorobiphenyl (HCB-153) (at 30, 100, 150 or 300 µmol/kg), or both compounds (at 30 or 100 µmol/kg). Rats were killed at selected time points, 3, 6, or 24 hours, 2, 6, or 10 days post PCB treatment, as indicated on the figures.

Livers were removed and 30% homogenates were prepared in ice-cold 0.25 M sucrose solution with 0.1 mM EDTA, pH 7.4. In vitro lipid peroxidation was determined colorimetrically by measuring the amount of malondialdehyde (thiobarbituric acid reactive substances, TBARS) in whole homogenates using the basic procedure of Uchiyama and Mihara (1978). The results are presented in the following two figures.

PCB compounds had no significant effects on TBARS formation with doses of 30, 150 µmol/kg in whole homogenates from rats treated for 6, 12, 24 hours, or 2, 6, or 10 days (first figure). However, significant increases in TBARS were observed 6 days post-treatment with TCB-77, HCB-153, and both TCB-77 and HCB-153 in rats receiving doses of 300, 300, and 100 of each, respectively (second figure). These results show that high concentrations of PCBs may result in oxidative stress in the liver. To analyze the occurrence of oxidative stress in the breast, a more sensitive endpoint was used, the measurement of 8-oxo-dG in breast tissue DNA (see below).

Effect of PCBs on Hepatic Whole Homogenate Lipid Peroxidation

Legend:
- Corn Oil
- TCB-77 30
- TCB-77 150
- HCB-153 30
- HCB-153 150
- Both PCBs (30+30)

Y-axis (vertical): Thiobarbituric Acid Reactive Substances (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45)

X-axis (categories): Time After Treatment — 3 Hours, 6 Hours, 24 Hours, 2 Days, 6 days, 10 days
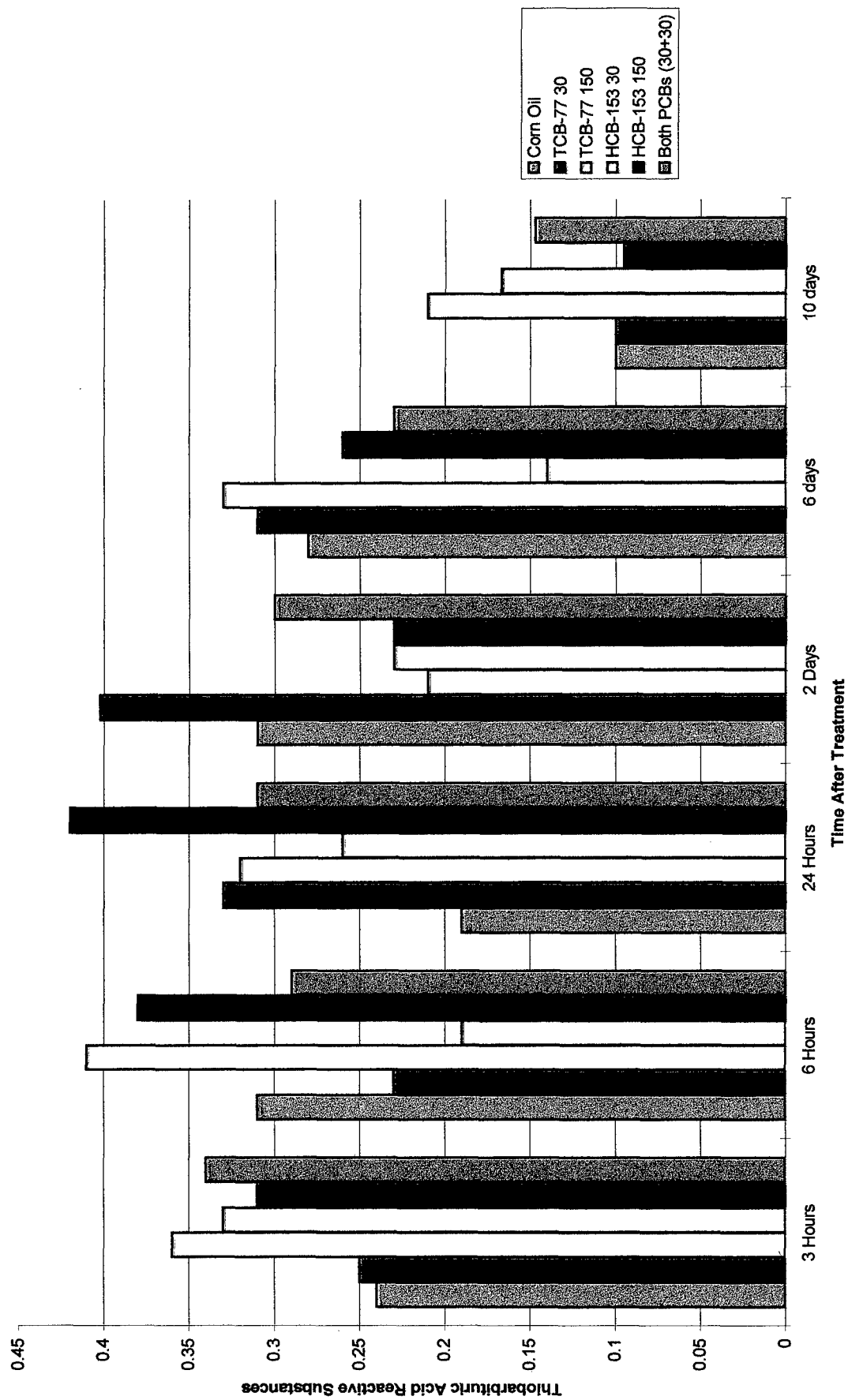
Effect of PCB Compounds on Hepatic Lipid Peroxidation

Thiobarbituric Acid Reactive Substances (nmol/mg Protein)

PCB Compounds

Legend: 2 Days, 6 days

X-axis labels: Corn Oil, 100 TCB-77, 300 TCB-77, 100 TCB-153, 300 TCB-153, 100+100 Both PCBs
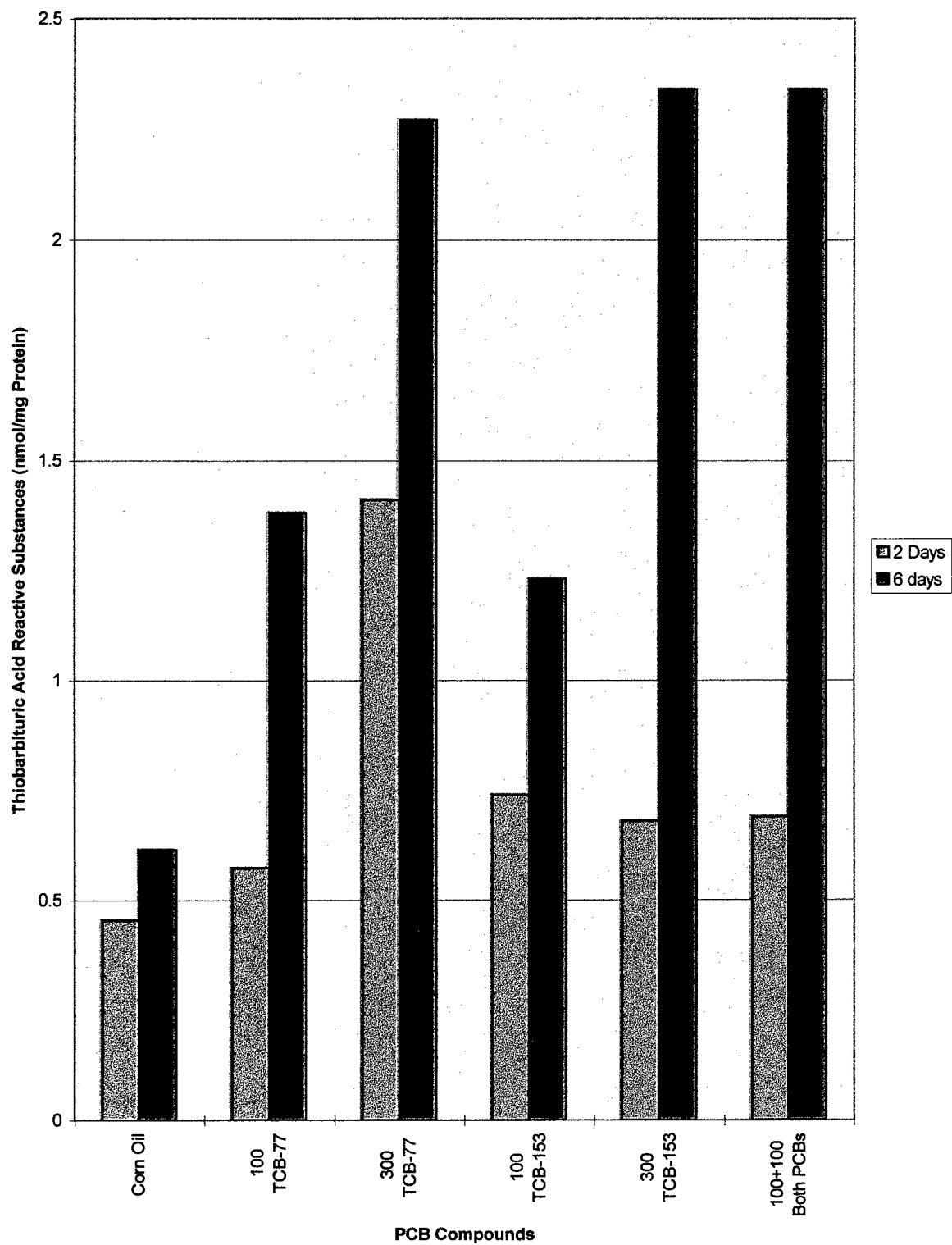
3. EFFECTS OF DI- AND TRICHLORO-BIPHENYLS ON THE MODULATION OF 8-OXO-2'-DEOXYGUANOSINE (8-oxodG) PRODUCTION IN RAT MAMMARY EPITHELIAL DNA

Standardization of DNA isolation procedure in order to minimize the artifactual production of 8-oxodG

There has been extensive debate lately over the tissue levels of 8-oxodG reported. It has been suggested that the levels may be artifactually increased during the DNA isolation process. The problem is further complicated when this lesion is measured by techniques such as GC-MS which involves derivatization at high temperature (Douki et al., 1996; Cadet et al. 1997). Studies reported in the past few months suggested that 8-oxodG levels could be substantially artifactually elevated during the DNA isolation unless non-phenol procedures were used (Helbock et al., 1998) or free radical traps were included throughout the DNA isolation process (Hofer & Moller, 1998). Our laboratory has also been engaged in major efforts to minimize artifactual production of 8-oxodG during DNA isolation.

DNA isolation procedure typically involves homogenization, enzymatic removal of RNA and proteins, DNA extractions with phenol, phenol:sevag and sevag, followed by ethanol precipitation. The artifactual production of 8-oxodG can be caused during the homogenization which may actually release several enzymes and transition metals (e.g. iron, Cu etc.) enough to initiate redox cycle. The enzymatic removal of RNA and proteins at 37°C could potentially accelerate the redox process if it was not protected with potent free radical traps such as antioxidants and/or metal chelators since some of these enzymes are reported to have oxidizing capability through their intrinsic transition metal cofactors. And finally, phenol could also oxidize DNA.

The experiments were designed with rat liver and DNA was isolated in the presence of a variety of antioxidants, 8-hydroxyquinoline (HQ) and BHT; metal chelators (EDTA) and electron spin trap, N-tert-butyl-α-phenylnitrone (PBN) in combination with room temperature or cold temperature (4°C) working conditions, addition of KCl to complex pro-oxidant enzymes in the presence of sodium dodecyl sulfate. The non-phenol DNA isolation procedures were also included for comparison (Wang et al., 1994).

The results suggest that the standard phenol procedure, the most commonly used DNA isolation procedure, resulted in a significantly higher values of 8-oxodG compared to the modified phenol procedure (28.7 ± 6.0 versus 16.4 ± 1.6 8-oxodG/10^6 nucleotides) (Table 1). Modified phenol procedure involves lower concentrations of RNAse A and T1, lower incubation times and substituting extraction with phenol alone with one more rounds of sevag extraction. The values were further reduced with the addition of either HQ or PBN alone; addition of KCl (40 mM) also seems to reduce 8-oxodG levels. However, the dramatic reduction (~85%) was observed with the combination of HQ, PBN and KCl at 4°C. On the other hand, BHT (5 and 30 mM) or guanine (3 and 10 mg/ml) alone or in combination with HQ (6.8 mM), added at each step or during phenol extractions did not reduce the 8-oxodG

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values (data not shown); BHT in combination with HQ, was also found ineffective. In the alternative non-phenol procedure, NaI (4.5 M) was used in combination with *iso*propanol to precipitate the DNA, while the other conditions remained constant in both the procedures. This procedure seems to result in 30-40% lower values of 8-oxodG than the standard phenol procedure. Again, 4°C working conditions reduced the values by about 40% (14.4 versus 21.5 8-oxodG/10^6 nucleotides). Further, HQ in combination with KCl resulted in a 3-fold reduction in the 8-oxodG values compared to the appropriate control at room temperature. Other electron spin traps like 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) alone or in conjunction with HQ or PBN by both phenol and non-phenol methods are presently being tested.

Table 1: 8-OxodG levels in rat liver DNA isolated by different methods[a]

Methods	8-oxodG/10^6 nucleotides	% of control
Experiment A:		
1. Standard Phenol[RT]	28.7 ± 6.0[b]	100
2. Modified Phenol[4°]	16.4 ± 1.6	57
3. Modified Phenol + BHT (10 mM)[*] + HQ (6.8 mM) during extractions[K, RT]	33.3 ± 4.5	116
4. Modified Phenol + HQ (6.8 mM)[K,**, RT]	11.7 ± 1.3	41
5. Modified phenol + HQ (6.8 mM)[* *, RT]	14.3 ± 0.6	50
6. Modified Phenol + PBN (100 uM)[**, 4°]	12.0 ± 1.9	42
7. Modified Phenol + HQ + PBN (100 uM)[K, **, 4°]	4.6 ± 0.6	16
8. Modified Phenol + HQ + PBN (100 uM)[K, **, RT]	10.5 ± 1.7	37
Experiment B:		
9. NaI method[RT]	21.5 ± 1.4	100
10. NaI method[4°]	14.4 ± 1.4	67
11. DNA (NaI method), followed by Standard Phenol extractions[RT]	34.4 ± 5.0	160
12. NaI method + BHT (10 mM)[K,*, RT]	16.6 ± 8.1	77
13. NaI method + HQ (6.8 mM)[K,*, RT]	7.4 ± 1.9	34

[a]Approximately 0.2 g tissue was processed for DNA isolation.
[b]Mean of 3 DNA preparations ± standard deviation, except in method 6 (n=2).
*BHT or HQ were added during tissue homogenization and RNAse treatment.
**HQ or PBN were added at each step including solvent extractions).
Modified phenol had 1x phenol:Sevag and 2x Sevag.
K = 40 mM KCl after proteinase K step to remove protein.
RT = room temp.

In conclusion, it appears that the major artifact-prone steps in the DNA isolation are the tissue processing and the enzymatic steps. Phenol and temperature may also contribute to certain extent to the artifactual production of 8-oxodG during DNA isolation.

Analysis of 8-oxodG in PCBs treated rat mammary tissues

As described above and in our recent publication (Gupta & Arif, 1998), the ^{32}P-postlabeling assay was significantly improved to quantify 8-oxodG in tissues. We then applied these improved techniques to measure 8-oxodG in rats after treatment with PCBs.

As above, female S/D rats (150-174 g) were pretreated with corn oil alone or a mixture of inducers, phenobarbital (PB) + β-naphthoflavone (β-NF) or higher chlorinated PCBs mixture) for three consecutive days, followed by three daily injections of either 3,4-dichlorobiphenyl (3,4-DCBP) or 3,4,5-trichlorobiphenyl (3,4,5-TCBP) intraperitoneally. The animals were euthanatized the following day by carbon dioxide asphyxiation. Mammary tissue was collected and stored at -80°C until DNA isolation. Mammary epithelial cells were harvested by collagenase treatment (Ip & Daniel, 1985). The antioxidant butylated hydroxy toluene (BHT) (5 mM) was added during the extractions to suppress any artifactual production of 8-oxodG by phenol. DNA (5-10 μg) was enzymatically digested and 8-oxodGp was enriched by TLC by removal of unadducted nucleotides (Gupta & Arif, 1998). The enriched 8-oxodGp was labeled with molar excess of [γ-^{32}P]ATP and T4 polynucleotide kinase, followed by 3'-dephosphorylation with nuclease P1. A 2-D PEI-cellulose TLC was used to separate 5'-^{32}P-labeled 8-oxodG followed by detection and quantification by Packard Instant Imager. The relative adduct labeling was calculated as [CPM$_{(8-oxodG)}$ ÷ CPM$_{normal\ nucleotides}$] x 1/dilution factor.

Table 2: Effect of PCBs treatment with or without inducers on 8-oxodG production in rat mammary tissue

Treatment	8-OxodG/10^6 nucleotides
	Mammary
Control	18.7 ± 1.9
Vehicle	31.2 ± 4.4
3,4-DCBP (100 μmol/kg)	18.7 ± 6.8
3,4,5-TCBP (100 μmol/kg)	8.4 ± 3.7
PB + ß-NF	12.9 ± 5.7
" + 3,4-DCBP (100 μmol/kg)	16.2 ± 4.2
" + 3,4,5-TCBP (100 μmol/kg)	22.3 ± 10.4
(3,4)^{2}Cl + (2,4,5)^{2}Cl	28.5 ± 12.5
" + 3,4-DCBP (100 μmol/kg)	20.0 ± 2.5
" + 3,4,5-TCBP (100 μmol/kg)	19.3 ± 5.9

Values represent mean ± SD of 6 animals except in control (n=3).
PB (80 mg/kg) and β-NF (100 mg/kg) while $(3,4)^2Cl$ and $(2,4,5)^2Cl$
were given 75 μmol/kg each as mixture in corn oil intraperitoneally.

Table 2 shows that the treatment of rats with 3,4-DCBP and 3,4,5-TCBP resulted in approximately 2 - 4 fold reduction in 8-oxodG levels in the mammary tissue. However, 8-oxodG levels were somewhat increased when the animals were pretreated with the cytochrome P450 inducers, PB + β-NF, as compared to the inducer alone. Interestingly, pretreatment with a mixture of higher chlorinated PCBs did not enhance the 8-oxodG levels, the values were in fact slightly decreased following treatment with 3,4-DCBP and 3,4,5-TCBP.

These results suggest a differential response of oxidative stress in the mammry tissue following exposure to the PCBs. In the real life scenario, humans are routinely exposed to several chemicals including PCBs, both lower and higher chlorinated forms which may produce a tissue targeted differential biological effects in the humans. Further studies are warranted to understand the role of PCB exposure in the metabolism-oriented biological effects of other chemical carcinogens in humans.

4. ANALYSIS OF SELENIUM–DEPENDENT GLUTATHIONE PEROXIDASE, GLUTATHIONE TRANSFERASE AND CATALASE ACTIVITIES IN <u>HUMAN</u> BREAST CYTOSOL

The present study was initiated in an attempt to measure baseline levels of the antioxidant enzymes, Selenium-dependent Glutathione Peroxidase (Se-GSHPx), Glutathione-S-Transferase (GST) and Catalase, in human breast cytosol. We were especially interested in the questions of age, race, and inter-individual variations of enzyme activities in the breast.

Of the normal human breast tissues on hand, an arbitrary decision was made to divide the individuals represented into four groups based on age and race. Six specimens were chosen at random belonging to each division. Group one consisted of tissue from older black females (OB), average age 40 years, the second group of older white females (OW), average age 47 years, while the third and forth groups were of younger black (YB), average age 20 years, and younger white females (YW), average age 24 years, respectively (Table 3).

Table 3: Four different groups of breast tissue cytosol

OB		OW		YB		YW	
Sample number	age in years	sample number	Age in years	Sample number	age in years	sample number	Age in years
1	39	18	43	3	25	15	24
9	39	20	58	4	22	17	29
11	41	34	47	5	18	22	17
12	42	35	46	6	19	28	27
13	42	38	44	8	17	29	27
21	38	39	45	14	20	32	21

Se–GSHPx activity was assayed with hydrogenperoxide as substrate according to the method of Paglia et al. (1967). The activity of GST was measured according to Jensson et al. (1974) with 1-chloro-2,4–dinitrobenzene as substrate while catalase activity was determined by the method of Beers and Sizer (1952).

Glutathione-Peroxidase (Se dependent) activity in human breast cytosol
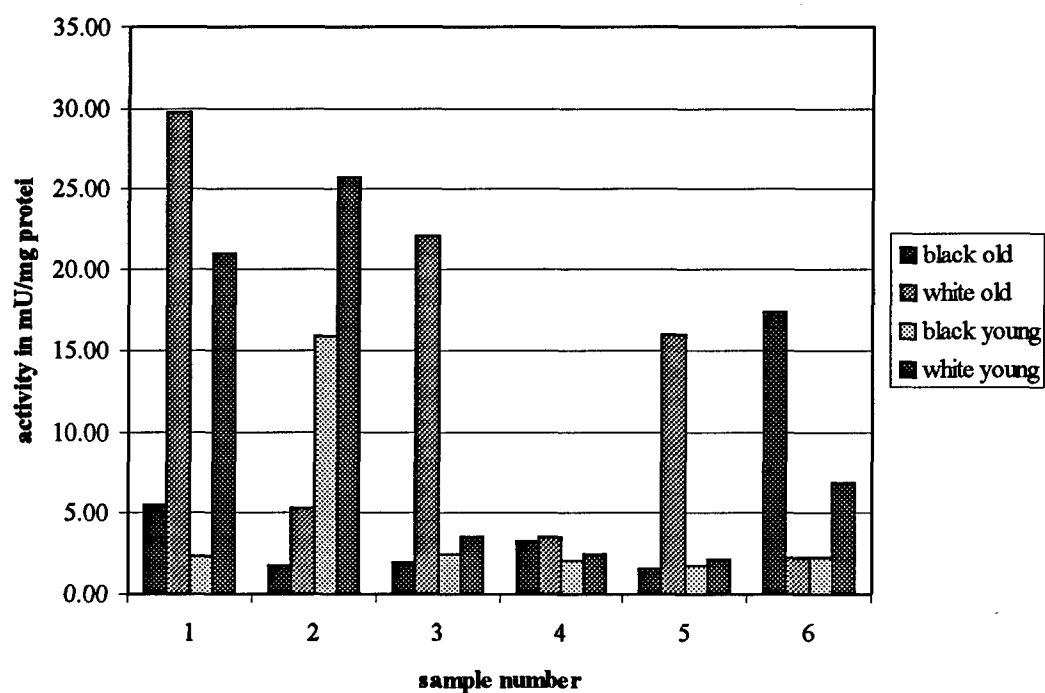
Glutathione - S - Transferase in human breast cytosol
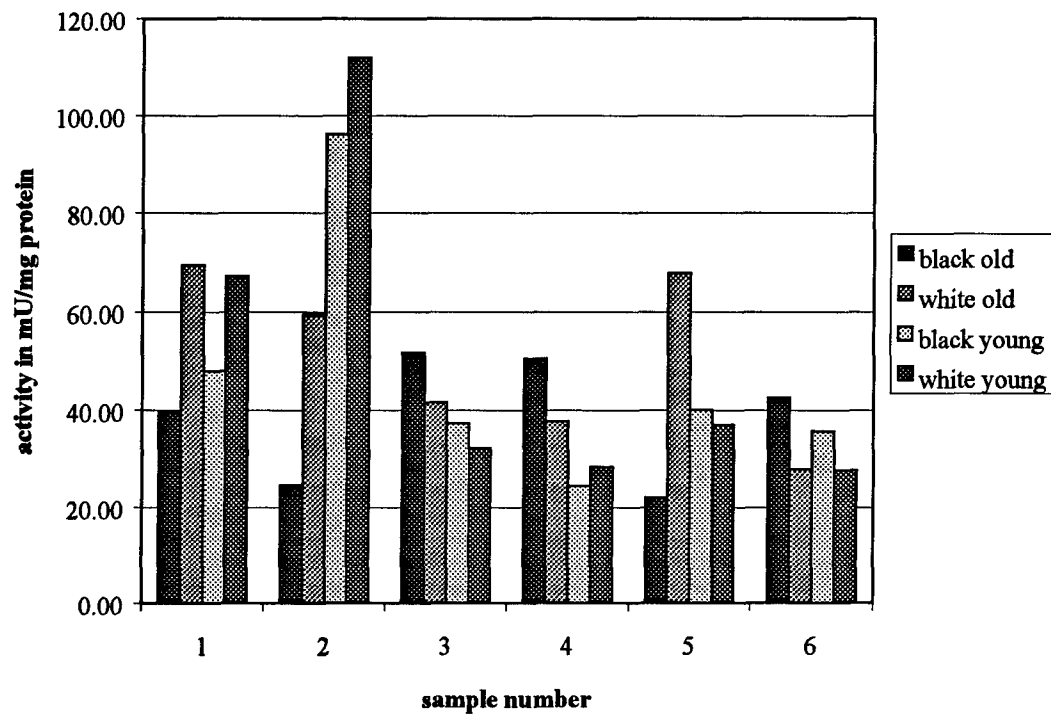
Catalase activity in human breast cytosol

These results show enormous inter-individual variations in activities of all three enzymes. The levels varied from 1.8 to 30 mU/mg protein for Se-GPX, from 22 to 112 mU/mg protein for GST, and from 1.7 to 124 U per mg protein for catalase. This may suggest a large inter-individual variation in susceptibility to damage by oxidative stress. No influence of age or race was detected.

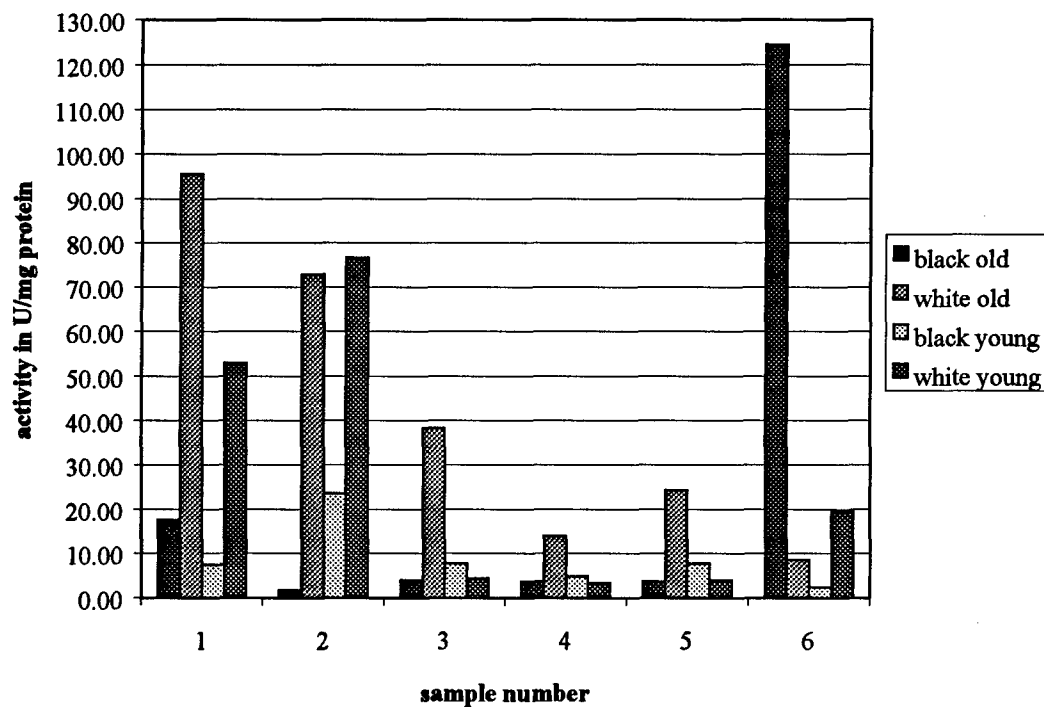These same human breast cytosols were employed to explore the possibility that the antioxidant enzymes present would inhibit or slow the redox cycling caused by PCB metabolites. A redox cycling reaction was set up exactly as described (McLean et al., 1998). The substrate used was 2-(3'-chlorophenyl)-1,4-benzoquinone, and the incubations were carried out for 1, 2, or 3 minutes, in the presence and absence of human breast tissue cytosols.
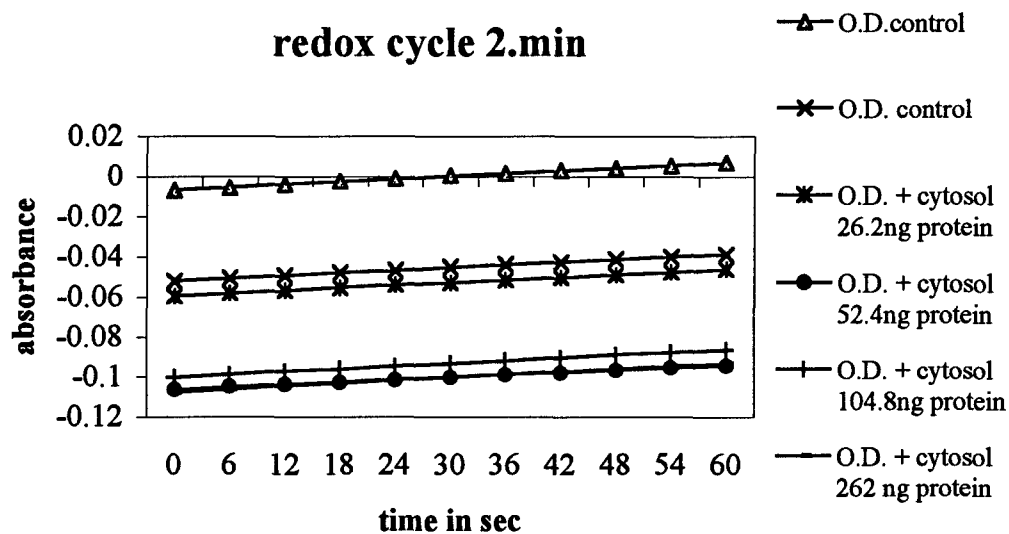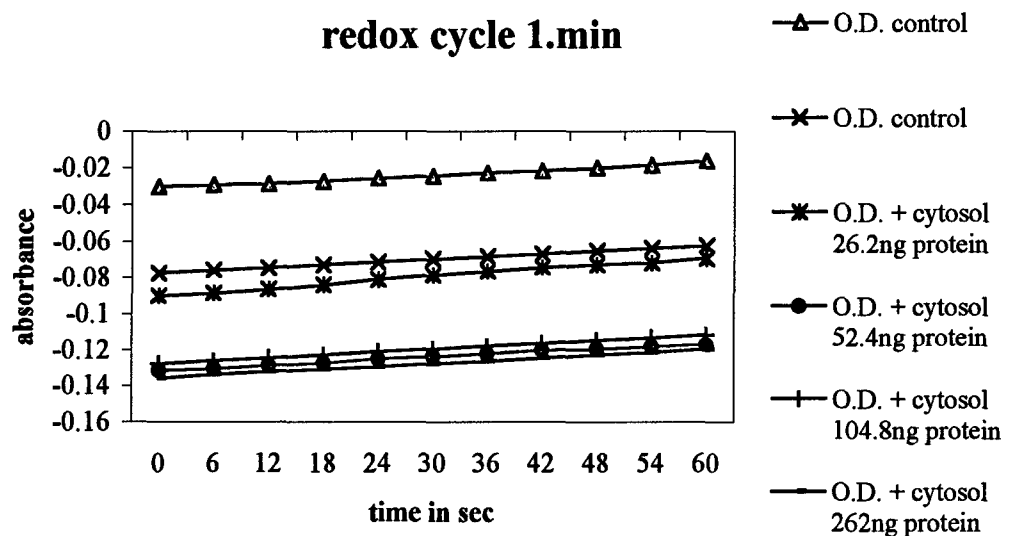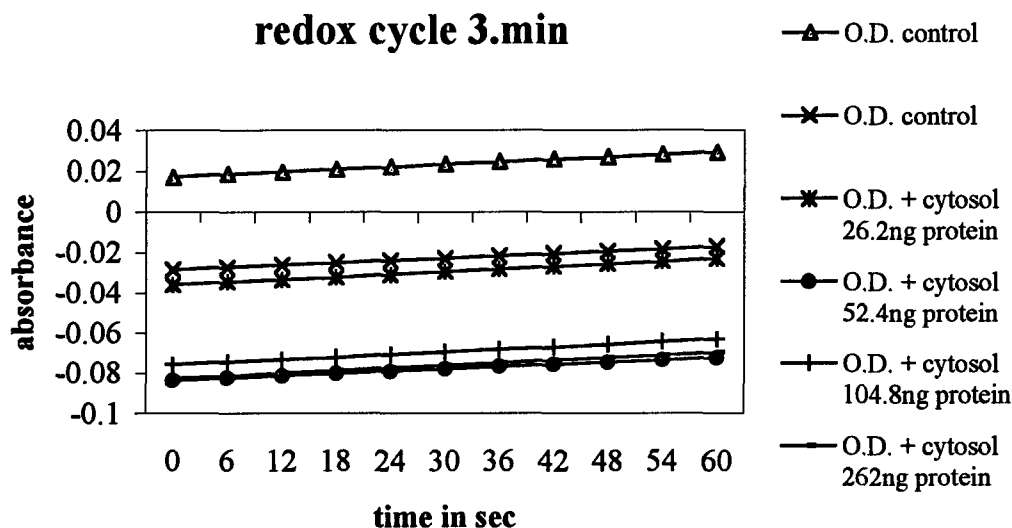
redox cycle 1.min

- O.D. control
- O.D. control
- O.D. + cytosol 26.2ng protein
- O.D. + cytosol 52.4ng protein
- O.D. + cytosol 104.8ng protein
- O.D. + cytosol 262ng protein

redox cycle 2.min

- O.D.control
- O.D. control
- O.D. + cytosol 26.2ng protein
- O.D. + cytosol 52.4ng protein
- O.D. + cytosol 104.8ng protein
- O.D. + cytosol 262 ng protein

redox cycle 3.min

There was no influence of any breast tissue cytosol on the rate of PCB-related redox cycling at any level of protein tested.

5. COMPARATIVE STUDIES OF ABILITY OF PCB METABOLITES TO SUSTAIN REDOX REACTIONS, PRODUCE REACTIVE OXYGEN SPECIES, INDUCE STRAND BREAKS AND CYTOTOXICITY

Studies to determine structure-activity relationships (SARs)

There are 208 possible chlorinated biphenyl isomers and an even larger number of possible metabolites. Obviously it is not feasible to test the activity of every one of them in every possible test. The more cost and time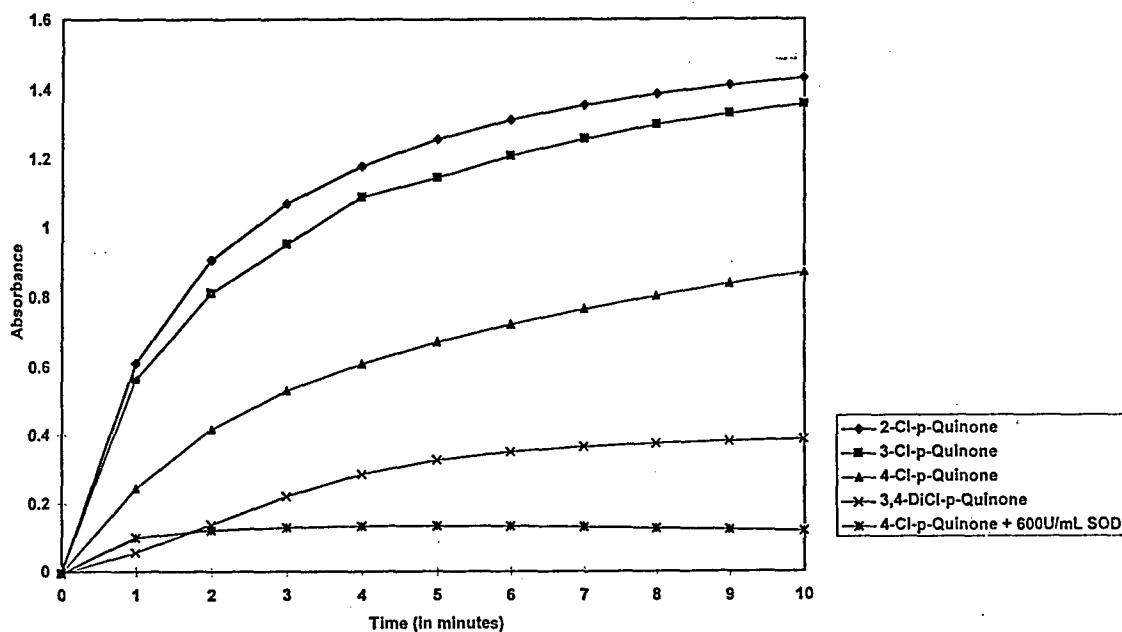 effective method is to analyze a subset of compounds to determine structure-activity relationships, which then allow to make informed predictions about the behavior of similar compounds. We therefore tested sets of dihydroxy-PCB metabolites which differ in the number and position of chlorines and/or position of the dihydroxy groups in various assays.

Using the NBT assay (reduction of nitrobluetetrazolium by superoxide to a blue formazan), we have shown previously that dihydroxy-PCBs can be oxidized by peroxidase/H_2O_2 to the corresponding quinone with production of superoxide. Ones the quinones are generated, the superoxide production ceases. When glutathione is added to the quinone, a dihydroxybiphenyl-glutathione conjugate is formed. We analyzed superoxide production in a system containing PCB-2,5-quinones and glutathione in equimolar concentrations. A formation of the blue formazan was observed, which shows that the conjugate can oxidize to the quinone-conjugate with production of superoxide. There was a clear influence of the position and number of chlorines on superoxide production with 2 > 3 > 4 > 3,4 (see the following figure). Even more superoxide is formed when more than 1 equivalent of GSH is added, due to multiple passes through the cycle of oxidation and reductive conjugation.

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Reduction of NBT by PCB-Quinones (100µM) in the presence of GSH (100µM)

Legend:
- 2-Cl-p-Quinone
- 3-Cl-p-Quinone
- 4-Cl-p-Quinone
- 3,4-DiCl-p-Quinone
- 4-Cl-p-Quinone + 600U/mL SOD

X-axis: Time (in minutes)
Y-axis: Absorbance

Induction of Strand Breaks by PCB Metabolites

solvent control

4-Cl-2',5'-quinone

4-Cl-3',4'-dihydroxybiphenyl

4-Cl-2',3'-dihydroxybiphenyl

3,5-Cl₂-2',5'-dihydroxybiphenyl

3,4-Cl₂-2',5'-dihydroxybiphenyl

4-Cl-2',5'-dihydroxybiphenyl

3-Cl-2',5'-dihydroxybiphenyl

2-Cl-2',5'-dihydroxybiphenyl

solvent control

supercoiled plasmid DNA
linear plasmid DNA
open circle plasmid DNA
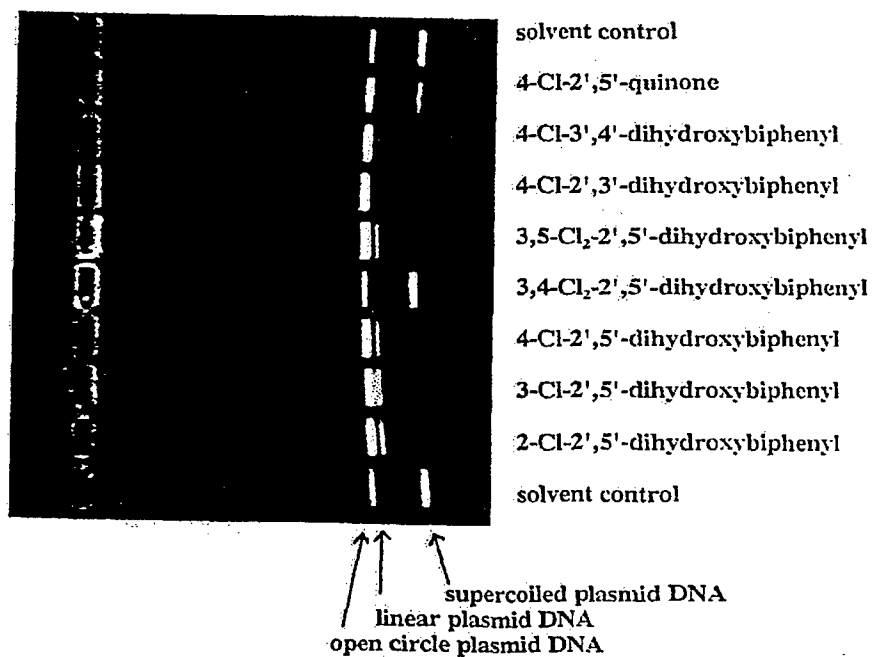
The chlorinated dihydroxybiphenyls were also compared in their ability to induce strand breaks. Undamaged plasmid DNA is in a supercoiled form. Single strand breaks result in open circle formation, and double strand breaks lead to linear DNA These 3 forms differ in their migration speed in agarose during gel electrophoreses and thereby can be separated. The following figure shows a gel with ethidium bromide stained DNA which was treated with different para-dihydroxy-PCBs, 4-Cl-2,5-quinone biphenyl, or solvent in the presence of Cu(II). Again an influence of the number and position of chlorines on the activity in the assay was observed. The strand breaking activity decreased in the order 3 > 2 > 4 = 3,5 > 3,4 chloro-2',5'-dihydroxybiphenyl. We also observed an effect of the position of the dihydroxy groups, with 2',5'- > 3',4'- > 2',3'-dihydroxy-4-Cl-biphenyl (data not shown).

One question was, whether the stronger activity in ROS and strand break production would result in an increased cytotoxicity of these compounds. Experiments with yeast (*Saccharomyces cerevisiae*) and bacteria (*E. coli*) showed us that the number and position of the chlorines of 2',5'-dihydroxybiphenyl changed the LD_{50} by more than 10x, resulting in an order of toxicity of 2- < 2,5- < 3- < 4- < 3,5- < 3,4-chloro- 2',5'-dihydroxybiphenyl (data not shown). This is, however, opposite the order of superoxide production in the NBT assay and strand break induction. Moreover, the order of 3-chloro-DHBP was 2',5'- < 2',3'- < 3',4'-dihydroxybiphenyl, again opposite to the order of superoxide and strand break production. This means that not the production of ROS, but some other factor is responsible for the cytotoxicity of these compounds.

Oxidation of dihydroxy biphenyls and binding to glutathione (GSH)

Recently several PCB metabolites were found in human serum, which had the dihydroxy groups in the 4,4'-position (Bergman et al., 1994). This confirms our hypothesis that dihydroxy PCBs are build in the human body and most importantly that these metabolites are surprisingly stable. We obtained a small amount of one of these metabolites, 3,5,3',5'-tetrachloro-4,4'-dihydroxybiphenyl, from one of the authors, Dr. A. Bergman (Sweden), to study oxidation and conjugation reactions. Before we used any of this valuable material, however, we wanted to know, how likely it is that such dihydroxy-biphenyls (DHBP) oxidize in aqueous solution, either non-enzymatically or enzymatically, and how easily the resulting quinone reacts with sulphydryl groups of proteins or peptides. We used 4,4'-DHBP to analyze oxidation and GSH conjugation and compared it with biphenyls which have OH-groups in 2,5-, 3,4-, and 2,2'- position.

Non-enzymatic oxidation of our test compounds was studied by measuring the spectrum before and after 6 hours incubation in phosphate buffer (PBS w/o Mg and Ca) at 37 C.   All DHBPs had a large peak in the UV spectrum at about 206-212nm, and a second or third (2,2'-DHBP) peak in the UV range of 260-310nm. No qualitative or remarkable quantitative changes in these spectra were seen after 6 hours with 3,4-, 2,2'- and 4,4'-DHBP, indicating that these compounds are stable under the assay conditions. The spectrum of 2,5-DHBP

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changed as described below, indicating a non-enzymatic oxidation of this compound.

Enzymatic oxidation was tested by incubating 100 µM compound with 1 U horseradish peroxidase (PRPx) and measuring the spectrum before and 1 min after the reaction was started with 500 µM H_2O_2.

- 3,4-DHBP: the peak at 260 nm disappeared, a new peak at 330 nm appeared;
- 2,5-DHBP: the peak at 300 nm disappeared, new peaks at 228, 247, and 376 nm appeared;
- 2,2'-DHBP: only minor changes in the spectrum were seen with HRPx/H_2O_2. When lactoperoxidase (LPx) was added the changes were more pronounced. The peak at 308 nm disappeared, a new peak at 418 appeared;
- 4,4'-DHBP: the peak at 260 disappeared, a new peak at 394 appeared. After the 4,4'-dihydroxybiphenyl was completely oxidized, a decrease of the peak in the visible region was observed. Also, a black precipitate was observed. The increase in unidentified metabolite(s), probably a polymerization product, caused a gradual drop of the absorption peak.

These changes indicate that all 4 compounds can be oxidized enzymatically with peroxidase/H_2O_2 to the corresponding quinone (BPQ), although the reaction is less pronounced with 2,2'-DHBP.

To test for binding with -SH groups an equimolar concentration of GSH (100 µM) was added to the DHBP/HRPx/H_2O_2 incubation and the spectrum determined. Then more GSH was added and the spectrum taken after each addition.

- 3,4-BPQ: a broad "peak/plateau" at about 300-330 nm appeared. Increasing the amount of GSH to 2x and 4x of the compound did not change the spectrum significantly.
- 2,5-BPQ: a peak appeared at 346 nm. Increasing the amount of GSH to 2x and 4x of the compound resulted in a disappearance of the 346 peak and a new peak at 334nm.
- 2,2'-BPQ: the peak at 418 disappeared.
- 4,4'-BPQ: the peak at 398 was reduced and a small new peak at about 249 appeared. By adding more equivalents of GSH (2x, 4x, 10x of compound), the 398 peak disappeared completely and the 249 peak increased and shifted slightly to 250, 252, and 254.

All 4 compounds showed changes in the spectrum indicative of GSH addition with reduction of the quinone to the dihydroxy-conjugate. With the 4,4'-derivative probably more than 1 equivalent of GSH was needed to achieve complete conjugation. The chemical reasons for the additional changes in the spectrum with the 2,5-compound could be that the conjugate oxidized non-enzymatically to the quinone and an additional 1 GSH was added to the molecule.

To summarize the results: only the p-DHBP oxidised non enzymatically, but all 4 compounds were oxidized by peroxidase/H_2O_2, although 2,2'-DHBP only reluctantly. Three of the resulting quinones conjugated GSH, with 4,4'- < 3,4- < 2,5-compound. GSH conjugation seems somewhat questionable with the 2,2'-metabolite.

4,4'-dihydroxy-3,3',5,5'-tetrachlorobiphenyl showed a UV band at λ = 231 and 302 nm, which is in agreement with the proposed structure. In the presence of HRP or lactoperoxidase, the colorless solution of the biphenyl in buffer turned immediately yellow after addition of hydrogen peroxide. The yellow color (λ = 434 nm) indicates the formation of the corresponding quinoid compound. The color started to fade after the addition of more hydrogen peroxide. In contrast to 4,4'-dihydroxybiphenyl, no black precipitate was observed. No quinone formation was observed in the presence of N-acetylcysteine, an observation which is in agreement with our earlier observations that N-acetylcysteine is added to the quinoid structure under re-aromatization (Scheme below).

Scheme 1

a: R = Cl
b: R = H

$$R\text{'-SH} =$$
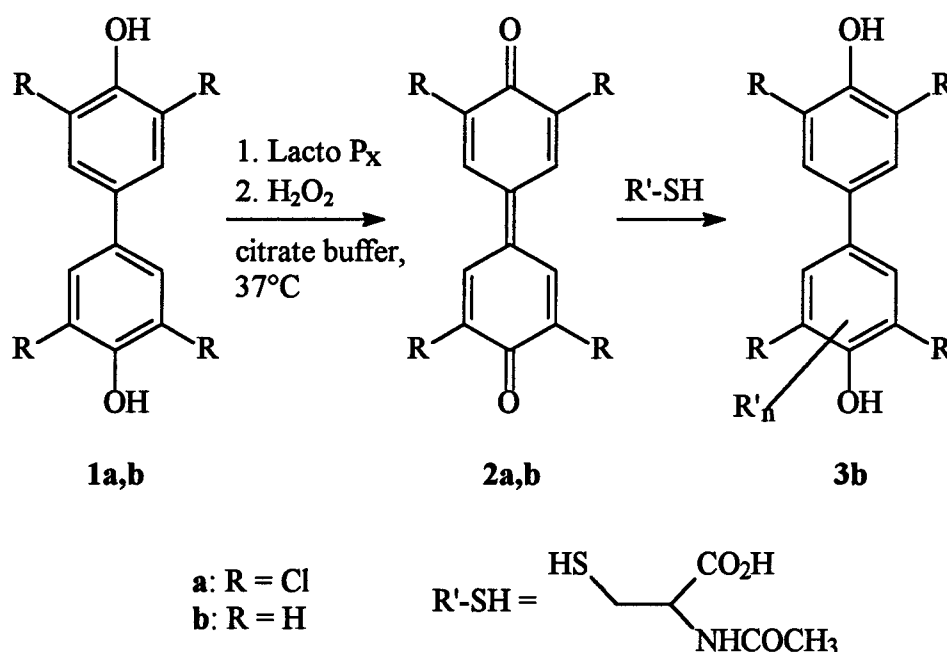
Scheme: Oxidation of 4,4'-Dihydroxybiphenyls in the Presence of Lactoperoxidase and Reaction with a S-Nucleophile (N-acetylcysteine)

Since all of our compounds were oxidized by peroxidase/H_2O_2 we expected that they all would result in superoxide production. When we tested 4,4'-DHBP and 2,2'-DHBP in the NBT assay, however, no formazan production could be observed, even though the quinone was formed as could be seen by taking the spectrum of the compound after enzyme treatment. At this point we are not able to explain this puzzling result. Further studies are planned to explain our observation.

In some preliminary experiments we also studied the oxidative effect of human breast cytosol on the oxidation of 4,4'-dihydroxy-3,3',5,5'-tetrachlorobiphenyl. This compound was chosen because it is easily oxidatized by peroxidases and should therefore be a sensitive indicator of oxidative activity in different cellular fractions. No oxidation of the dihydroxybiphenyl compound

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was observed in three samples (# 28, young white; #32, young white; #38, old white). When HRP was added as a control, the test compound was oxidized immediately. This indicates that there is neither an oxidizing component nor anything that inhibits peroxidase mediated oxidation in human breast cytosol.

Cytotoxicity of hydroxylated biphenyls

Dihydroxybiphenyls (DHBP) with hydroxyl groups in 2,5-, 3,4-, 2,2'-, and 4,4'- position were tested for cytotoxicity in several cell lines, among them the MCF-7, MDA-MD-231, and T47D breast carcinoma, LNCap prostate carcinoma, and HMEC human microvascular endothelial cell line. Cells were exposed to the test compounds for 3 days after which the percentage of cells compared to untreated controls were determined with the MTT assay. The sensitivity of the different cell lines to the test compounds varied, but no trend could be observed. In general the order of toxicity was 2,5- > 3,4- > 4,4'- > 2,2'-DHBP, with an LD_{50} in the range of 15-55 µM, 12-120 µM, 30-200 µM, and 90-600 µM, respectively. This order seems to correlate with the ease of oxidation and GSH adduct formation. Oxidation followed by protein binding could therefore be the major cause of cell death, especially with the 2,5-, 3,4- and 4,4'-DHBPs. Peroxidase mediated oxidation and binding to protein and peptide -SH groups was reported for 4,4'-DHBP (McGirr et al., 1986). 2,2'-DHBP was shown to be oxidized by peroxidase/H_2O_2, but no or little covalent binding to protein or peptide -SH groups was observed (Subramanyam et al., 1990).

When peroxidase activity is a prerequisite for oxidation and toxicity of compounds which do not oxidize spontaneously in aqueous solution than cells with intracellular peroxidase should be more threatened by these compounds than cells without intracellular peroxidase. We therefore tested the cytotoxicity of the above compounds in HL60 cells, human promyelocytic leukemia cells, which contain intracellular peroxidase. The LD_{50} after 3 days of exposure was about 1 µM for 2,5-, 2.5 µM for 3,4-, 5-15 µM for 4,4'-, and 50 µM for 2,2'-DHBP. This means that this cell line was significantly more sensitive to all 4 compounds than the above mentioned cell lines. Whether this is due to the intracellular peroxidase or due to the fact that this was the only non-adherened growing cell line remains to be determined. HL60R, an HL60 clone that can no longer be induced to differentiate, was tested in parallel. Surprisingly, these cells were repeatedly about 4x less sensitive to 2,5- and 3,4-DHBP, but showed the same sensitivity to 2,2'- and even slightly more sensitivity to 4,4'-DHBP. The reason for this difference remains to be determined. Also, bacteria (*E. coli* strain JM109) were not negatively affected by 4,4'-DHBP up to a concentration of at least 1 mM; they even seemed to thrive in medium with 4,4'-DHBP.

One of the metabolites which were recently discovered in human serum, 3,5,3',5'-tetrachloro-4,4'-dihydroxybiphenyl (TC-DHBP), was also tested for cytotoxicity induction in MCF-7 human breast cancer cells. Unlike its unchlorinated relative, 4,4'-DHBP, this compound was not toxic to MCF-7 cells up to a concentration of 500 µM (1 day exposure).

The toxicity of the 2',5'- and 3',4'-dihydroxy-metabolites of 3,4-dichlorobiphenyl was determined using HL60 peroxidase containing and Jurkat

cells, which have no intracellular peroxidase. The cytotoxicity of these compounds after 24 hours of exposure was so similar in the 2 different cell lines, with an LD_{50} of about 3 µM for the 2',5'- and 25-45 µM for the 3',4'-metabolite, that we cannot deduct a strong influence of intracellular peroxidase activity from these results.

6. ANALYSIS OF DNA ADDUCTS BY HPLC

Highly reactive chemicals, for example quinoid metabolites of environmental contaminants, can covalently bind to cellular DNA. Adducts that are not removed from the DNA by cellular defense mechanisms can lead to mutations that are transferred to future generations of cells. A series of subsequent events can lead to cellular transformation and carcinogenesis. There is currently great interest in DNA adducts as biomarkers of carcinogen exposure. The measurement of DNA adducts has important applications for cancer risk assessment.

The goal of our work is to prepare adducts of quinoid PCB metabolites with DNA. We studied the formation of DNA adducts of these quinones and the individual nucleosides or nucleotides in order to determine their structure and to optimize the HPLC separation. Additionally, we developed an assay to isolate adducts after enzymatic digestion of adducted DNA.

The formation of DNA adducts of benzoquinone with 2'-deoxycytidine and 2'-deoxyadenosine was described recently. Similar DNA adducts may be formed by PCB quinones. In some preliminary experiments, we studied the formation of DNA adducts with 2-(2-chlorophenyl)-1,4-benzoquinone with 2'-deoxycytidine, 2'-deoxythymidine, 2'-deoxyguanosine and 2'-deoxyadenosine. 2'-Deoxycytidine does not form a DNA adduct in a suspension of 2-(2-chlorophenyl)-1,4-benzoquinone (0.1 M sodium acetate buffer, pH 4.5, 20°C) because the chlorinated phenyl-benzoquinones are insoluble in water. A similar reaction was performed in methanol because of this solubility problem. HPLC analysis showed four major products, some of which could be DNA adducts. The separation and characterization of these products is currently under way. We plan to synthesize DNA adducts with nucleotides after optimization of the reaction conditions and make them available as analytical standards for postlabeling experiments.

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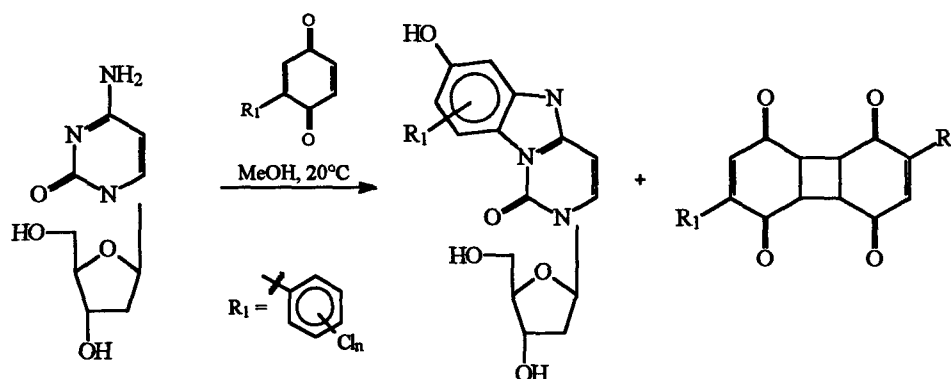
Scheme 3: Reaction of 2'-deoxycytidine with benzoquinones

Parallel, an assay for the digestion of (adducted) DNA and an HPLC methodology for the separation of the obtained nucleotides was developed: Calf thymus DNA (Sigma, St. Louis, MO) (160 mg) was suspended in STE buffer (25 mL) by stirring overnight. A 1.5 mL aliquot was transferred to a 15 mL plastic conical vial and the sample immersed in boiling water for 15 min to denature. The sample was rapidly cooled and to it was added nuclease P1 (40 μL, ~12U), 40 mM $ZnSO_4$ (4.0 μL) and 3 M sodium acetate (8.0 μL). The resultant sample was vortex mixed and immersed in a 65 °C shaker bath for 60 min. The sample was again treated with nuclease P1 and returned to the bath for an additional hour. Repeated enzyme treatments were required to digest the DNA into monomeric nucleotides. A 150 μL aliquot was removed and to it was added 850 uL of phosphatase buffer. To the sample was added alkaline phosphatase (2 μL, ~6U), the sample mixed and immersed in a 37°C water bath for 30 min. This treatment was repeated once. This procedure in theory affords monomeric dephosphorylated nucleosides.

HPLC analyses were performed on a Shimadzu system consisting of a SCL-10A system controller, 2 LC-10AS liquid chromatograph pumps, SPD-10A UV-vis detector, SIL-10A auto injector and CR501 Chromatopac data module. The column used is a Supelcosil LC-18S 25 mm x 4.6 mm I.D., 5 μm particle size C-18 (Supelco, Bellefonte, PA) All solvents were 0.2 μm filtered and degassed under reduced pressure before use. Final chromatographic conditions were: 1.0 mL/min flow rate, linear gradient from 100% 50 mM NaH_2PO_4(aq., pH 4.5) to 60:40 NaH_2PO_4:MeOH in 40 min, at ambient temperature with a 10 μL injection volume. Standards of the nucleosides 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine and thymidine (1.0 mg/mL, aqueous) were injected to determine their retention times. Enrichment of the digested DNA sample with the individual nucleosides was used to verify their identity.

These studies are just beginning and will be expanded in the next phase of this grant.

CONCLUSIONS:

1. Higher chlorinated PCBs (#77+153) *i.* induce cytochromes P450s, *ii.* result in an increase in GST activity, *iii.* lead to a decrease in catalase activity levels, and *iv.* result in decreased GSH levels. These changes may be the result and/or the reason for increased oxidative stress. These data were obtained from the liver samples of the treated animals. Analysis of these parameters in breast tissue is in progress.

2. PCB compounds had no effect on TBARS formation (indicator of lipid peroxidation/oxidative stress) with doses of 30, 150 μmol/kg in whole homogenates from rats treated for 6, 12, 24 hours, or 2, 6, or 10 days. However, significant increases in TBARS were observed 6 days post-treatment with TCB-77, HCB-153, and both TCB-77 and HCB-153 in rats receiving doses of 300, 300, and 100 of each, respectively. These results show that high concentrations of PCBs may result in oxidative stress in the liver. To analyze the occurrence of oxidative stress in the breast, a more sensitive endpoint was used, the measurement of 8-oxo-dG in breast tissue DNA (see below).

3. Methods development for 8-oxo-deoxy-guanosine determination in DNA showed that the major artifact-prone steps in the DNA isolation are the tissue processing and the enzymatic steps. Phenol and temperature may also contribute to certain extent to the artifactual production of 8-oxodG during DNA isolation. Our results, obtained with our improved method, suggest a differential response of oxidative stress in the mammary tissue following exposure to the PCBs. In the real life scenario, humans are routinely exposed to several chemicals including PCBs, both lower and higher chlorinated forms which may produce a tissue targeted differential biological effects in the humans. Further studies are warranted to understand the role of PCB exposure in the metabolism-oriented biological effects of other chemical carcinogens in humans

4. The results of our measurements of antioxidant enzyme activities in human breast tissue cytosols show enormous inter-individual variations in activities of all three enzymes investigated. The levels varied from 1.8 to 30 mU/mg protein for Se-GPX, from 22 to 112 mU/mg protein for GST, and from 1.7 to 124 U per mg protein for catalase. This may suggest a large inter-individual variation in susceptibility to damage by oxidative stress. No influence of age or race was detected. There was no influence of any breast tissue cytosol on the rate of PCB-related redox cycling at any level of protein tested.

5.
a. During the oxidation of dihydroxybiphenyls, the formation of the blue formazan was observed, showing the production of superoxide. There was a clear influence of the position and number of chlorines on superoxide production with 2 > 3 > 4 > 3,4. Even more superoxide is formed when more than 1 equivalent of GSH is added, due to multiple passes through the cycle of oxidation and

reductive conjugation.

b. Again an influence of the number and position of chlorines on the activity of PCB metabolites in causing stand breaks was observed. The strand breaking activity decreased in the order 3 > 2 > 4 = 3,5 > 3,4 chloro-2',5'-dihydroxybiphenyl. We also observed an effect of the position of the dihydroxy groups, with 2',5'- > 3',4'- > 2',3'-dihydroxy-4-Cl-biphenyl.

c. In contrast, the cytotoxicity experiments with yeast (<u>Saccharomyces cerevisiae</u>) and bacteria (E. coli) showed us that the number and position of the chlorines of 2',5'-dihydroxybiphenyl changed the LD_{50} by more than 10x, resulting in an order of toxicity of 2- < 2,5- < 3- < 4- < 3,5- < 3,4-chloro- 2',5'-dihydroxybiphenyl.

d. We investigated the propensity of a dihydroxy compound to be oxidized to a two-ring quinone. The p-dihydroxybiphenyl oxidised non enzymatically, whereas this compound and all one-ring quinone precursors examined were oxidized by peroxidase/H_2O_2, although 2,2'-DHBP only reluctantly. Three of the resulting quinones conjugated GSH, with 4,4'- < 3,4- < 2,5-compound.

e. In some preliminary experiments we also studied the oxidative effect of human breast cytosol on the oxidation of 4,4'-dihydroxy-3,3',5,5'-tetrachlorobiphenyl. This compound was chosen because it is easily oxidatized by peroxidases and should therefore be a sensitive indicator of oxidative activity in different cellular fractions. No oxidation of the dihydroxybiphenyl compound was observed in three samples (# 28, young white; #32, young white; #38, old white). When HRP was added as a control, the test compound was oxidized immediately. This indicates that there is neither an oxidizing component nor anything that inhibits peroxidase mediated oxidation in human breast cytosol.

f. One of the metabolites which were recently discovered in human serum, 3,5,3',5'-tetrachloro-4,4'-dihydroxybiphenyl (TC-DHBP), was also tested for cytotoxicity induction in MCF-7 human breast cancer cells. Unlike its unchlorinated relative, 4,4'-DHBP, this compound was not toxic to MCF-7 cells up to a concentration of 500 µM (1 day exposure).

6. Preliminary studies have been undertaken to react PCB metablites with DNA bases and to develop the appropriate HPLC conditions to isolate and characterize the reaction products.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)   23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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2. Point of contact for this request is Ms. Judy Pawlus at
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FOR THE COMMANDER:

Encl   PHYLIS M. RINEHART
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